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The effect of aristolochic acid I on expression of NAD(P)H:quinone oxidoreductase in mice and rats – a comparative study

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Abbreviations: AA, aristolochic acid; AAI, aristolochic acid I; AAIa, aristolochic acid Ia; AAN, AAII, aristolochic acid II; aristolochic acid nephropathy; BEN, Balkan endemic nephropathy; CYP, cytochrome P450; dA-AAI, 7-(deoxyadenosin-N^6-yl)-aristolactam I; dG-AAI, 7-(deoxyguanosin-N^2-yl)aristolactam I; dA-AAI, 7-(deoxyadenosin-N^6-yl)aristolactam II; HRN, Hepatic P450 Reductase Null; IARC, International Agency for Researcfh on Cancer; NQO1, NAD(P)H:quinone oxidoreductase; POR, NADPH:P450 oxidoreductase; RAL, relative adduct labeling; SDS, sodium dodecyl sulphate; S.E.M., standard error of the mean; TLC, thin-layer chromatography; WT, wild-type; thin-layer chromatography.

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Abstract

Aristolochic acid is the cause of aristolochic acid nephropathy (AAN) and Balkan endemic nephropathy (BEN) and their associated urothelial malignancies. Using the Western blotting, we investigated expression of NAD(P)H:quinone oxidoreductase (NQO1), the most efficient cytosolic enzyme that reductively activates aristolochic acid I (AAI), in mice and rats. In addition, the effect of AAI on NQO1 protein expression and its enzymatic activity in these experimental animal models was examined. We found that NQO1 protein levels in cytosolic fractions isolated from liver, kidney and lung of mice differed from those expressed in these organs of rats. In mice, the highest levels of NQO1 protein and NQO1 activity were found in the kidney, followed by lung and liver. In contrast, the NQO1 protein levels and enzyme activity were lowest in rat kidney cytosol, whereas the highest amounts of NQO1 protein and activity were found in lung cytosols, followed by those of liver. NQO1 protein and enzyme activity were induced in liver and kidney of AAI-pretreated mice compared with those of untreated mice. NQO1 protein and enzyme activity were also induced in rat kidney. Furthermore, the increase in hepatic and renal NQO1 enzyme activity was associated with AAI bioactivation and elevated AAI-DNA adduct levels were found in *ex vivo* incubations of cytosolic fractions with DNA and AAI. In conclusion, our results indicate that AAI can increase its own metabolic activation by inducing NQO1, thereby enhancing its own genotoxic potential.

1. Introduction

The herbal drug aristolochic acid (AA) derived from *Aristolochia* species has been shown to be the cause of so-called Chinese herbs nephropathy (CHN), now termed aristolochic acid nephropathy (AAN) [1,2]. The plant extract AA is a mixture of structurally related nitrophenanthrene carboxylic acids, the major components being aristolochic acid I (AAI) (Fig. 1) and aristolochic acid II (AAII). AAN is a rapidly progressive renal fibrosis that was initially observed 20 years ago in a group of Belgian women who had ingested slimming pills containing *Aristolochia fangchi* [3,4]. Within a few years of taking the pills, AAN patients also showed a high risk (~50%) of upper urothelial tract carcinoma and, subsequently, bladder urothelial carcinoma. In the meantime, it has become clear that AAN is a world-wide environmental and iatrogenic disease associated with urothelial cancer in humans [2,5,6]. Dietary exposure to AA has also been linked to Balkan endemic nephropathy (BEN) and its associated urothelial cancer [6-8]; this nephropathy is endemic in certain rural areas of Serbia, Bosnia, Croatia, Bulgaria and Romania.

Exposure to AA was demonstrated by identification of specific AA-DNA adducts in renal tissue of AAN and BEN patients [2,5-13]. The most abundant DNA adduct detected in patients is 7-(deoxyadenosin-N^6-yl)-aristolactam I (dA-AAI) (Fig. 1), which causes characteristic AT→TA transversions. Such an AT→TA mutational signature was predominant in the *TP53* tumor suppressor gene of urothelial tumours from AAN and BEN patients [4,8,14,15], a class of mutations accounting otherwise for only approximately 5% of all the *TP53* mutations in non-AA-associated human urothelial tumours according to the International Agency for Research on Cancer (IARC) TP53 database [16]. The same AT→TA transversions have been induced experimentally in human *TP53* in mouse embryonic fibroblasts from Hupki (Human *TP53* knock-in) mice treated *in vitro* with AAI [17,18] thus indicating a probable molecular mechanism associated with AA-induced carcinogenesis

[6,19]. AA has been classified as a Group I carcinogen in humans by IARC [20]. The National Toxicology Program has listed AA *as known to be a human carcinogen* for the first time in its 12th Report on Carcinogens.

The activation pathway for AAI is nitroreduction, catalyzed by both cytosolic and microsomal enzymes; in this process NAD(P)H:quinone oxidoreductase (NQO1) is the most efficient cytosolic nitroreductase [21-26] (Fig. 1). In contrast to NQO1, we found that conjugation enzymes such as human sulfotransferases or *N,O*-acetyltransferases did not significantly activate AAI [21,25,26]. AAI is also activated by microsomal enzymes; human, rat and mouse cytochrome P450 (CYP) 1A1 and 1A2 reductively activate AAI to form DNA adducts both *in vitro* and *in vivo* [27-33]. Beside NQO1 and CYP1A1/2, microsomal NADPH:P450 oxidoreductase (POR) also activates AAI, but plays a minor role [32,33]. Human and rodent CYP1A1 and 1A2 are also the principal enzymes involved in oxidative detoxication of AAI to the *O*-demethylation metabolite 8-hydroxyaristolochic acid I (aristolochic acid Ia, AAIa, Fig. 1) *in vitro* and *in vivo* [34-37]. Previous studies also suggested that, in addition to CYP1A1 and 1A2 expression levels, oxygen concentrations in specific organs or even cells might affect the balance between AAI nitroreduction (activation) and demethylation (detoxication) which, in turn, would influence tissue-specific toxicity or carcinogenicity of AA [22,23,27-30]. However, reductive activation of AAI in organisms may not only be dictated by CYP1A1/2, but also by the expression of the major AAI activating enzyme, NQO1. Indeed, we observed that higher AAI-DNA adduct levels in *Cyp1a1(−/−)* and *Cyp1a2(−/−)* single-knockout and *Cyp1a1/1a2(−/−)* double-knockout mouse lines [29] relative to wild-type (WT) mice were not only the result of lack of AAI demethylation by the CYP-dependent system, but also by higher NQO1 activity, which activates AAI [27,29,30,38]. Similar results were obtained in Hepatic P450 Reductase Null (HRN) mice, in which the *Por* gene is deleted specifically in hepatocytes, resulting essentially in the absence

of CYP activity in the liver [27]. Expression of NQO1 protein in hepatic and/or renal cytosolic samples was higher in several of these mouse lines compared to WT mice [27,38]; this was paralleled by increased NQO1 activity and AAI-DNA adduct formation in *ex vivo* cytosolic incubations with DNA and AAI. Collectively, these results suggested that deletion of *Cyp1a1/2* or *Por*, and therefore the absence of these enzymes, is partially compensated by increased expression of the cytosolic nitroreductase NQO1 [27,29,30].

Because of the importance of NQO1 in reductive activation of AAI, the aim of this study was to asses NQO1 protein expression and the effect of AAI on this expression in two animal models. As mouse and rat models of AAN are now currently used in standardized experimental protocols, we utilized them for such a study. Using the Western blotting we measured the expression levels of this enzyme in hepatic, renal and pulmonary cytosols isolated from rats and mice either untreated (control) or treated with AAI. In addition, NQO1 enzyme activity and DNA adduct formation in *ex vivo* cytosolic incubations with DNA and AAI were measured in these subcellular fractions.

2. Material and Methods

2.1. Animal experiments

Age-matched C57BL/6J mice, purchased from The Jackson Laboratory (Bar Harbor, ME, USA), were the same as those used in our previous studies [29,30]. Groups of female mice (3 months old; 25-30 g; n = 4/group) were treated with a single dose of AAI as sodium salt in water (50 mg/kg body weight) by oral gavage as described previously [29,30]. Control mice received water only. Animals were killed 24 h after the last treatment.

Groups of male Wistar rats (~150 g, n = 4/group) were treated with a single *i.p.* injection of AAI dissolved in 1% $NaHCO_3$, at a dose of 20 mg/kg body weight. Control rats receive the solvent only. Animals were killed 24 h after the last treatment. The study was conducted in accordance with the Regulations for the Care and Use of Laboratory Animals (311/1997, Ministry of Agriculture, Czech Republic), which is in compliance with the Declaration of Helsinki.

2.2. Preparation of cytosolic samples

Hepatic, renal and pulmonary cytosolic fractions from untreated and AAI-pretreated mice and rats were isolated as previously described [27-30]. Pooled cytosolic fractions (n = 4 mice[rat]/group] were used for subsequent analyses.

2.3. Determination of NQO1 protein levels by Western blotting

NQO1 antibodies were prepared as described previously [39]. Immunoquantification of cytosolic NQO1 was carried out on proteins transferred to a polyvinylidene fluoride membrane after separation by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis [39-41]. Human recombinant NQO1 (Sigma) was used to identify the NQO1

band from mouse and rat cytosols. Glyceraldehyde phosphate dehydrogenase was used as loading control and detected by its antibody (1:750, Millipore; MA, USA).

2.4. Measurement of NQO1 enzyme activity

NQO1 activity was determined using menadione as a substrate, as described by Ernster [42] but improved by addition of cytochrome *c* [43].

2.5. Cytosolic AAI-DNA adduct formation by ^{32}P-postlabeling

Incubation mixtures, in a final volume of 750 µl, included 50 mM Tris-HCl buffer (pH 7.4) containing 0.2% Tween 20, 1 mM NADPH, 1 mg mouse and rat cytosolic protein, 0.5 mg calf thymus DNA (2 mM dNp) and 0.5 mM AAI [21]. Incubations were carried out at 37°C for 60 min; AAI-derived DNA adduct formation is known to be linear up to 2 hr [25]. Control incubations were carried out (*i*) without cytosol, (*ii*) without NADPH, (*iii*) without DNA, or (*iv*) without AAI. After extraction with ethyl acetate, DNA was isolated from the residual water phase by the standard phenol/chloroform extraction method. ^{32}P-Postlabeling assay using the nuclease P1 enrichment version, and thin-layer chromatography (TLC) for analysis of AAI-DNA adduct formation, were performed as described [10,22]. TLC sheets were scanned using a Packard Instant Imager (Dowers Grove; USA). DNA adduct levels (RAL, relative adduct labeling) were calculated as described [11,21,25]. Results were expressed as DNA adducts/10^8 nucleotides.

3. Results

Using Western blotting, we investigated the organ specificity of NQO1 protein expression and the effects of AAI treatment on its expression in two experimental animal models, mice and rats. Liver, kidney and lung cytosolic samples isolated from control (untreated) animals and AAI-pretreated animals were used for analyses.

3.1. Determination of NQO1 protein expression and evaluation of NQO1 enzyme activity in the liver, kidney and lung of mice and rats

Expression of NQO1 protein was detected in all cytosolic samples in both animal models. Expression levels of this enzyme were different in the tissues (organs) investigated, both in mice and rats. In mice, the highest expression of NQO1 protein was found in kidney; levels were 2.5- ($p<0.001$) and 1.3-fold ($p<0.05$) higher in kidney and lung relative to liver, respectively (Fig. 2A, *upper panel*). Likewise, the highest NQO1 activity, measured with menadione as a substrate, was found in mouse kidney, followed by lung and liver (Fig. 2A, *lower panel*).

Interestingly, in rats the highest NQO1 protein levels were observed in lung, followed by liver and kidney; levels were 2.0- ($p<0.001$) and 1.3-fold ($p<0.05$) higher in pulmonary and hepatic cytosols relative to renal cytosols, respectively (Fig. 2B, *upper panel*). NQO1 protein levels in individual rat organs were paralleled by the specific activity of this enzyme; NQO1 activity was 3.2- and 1.6-fold (both $p<0.001$) higher in lung and liver cytosols relative to cytosols of kidney, respectively (Fig. 2B, *lower panel*).

3.2. The effect of AAI pretreatment on NQO1 protein expression and NQO1 enzyme activity in liver, kidney and lung of mice and rats

Pretreatment of mice with AAI increased NQO1 protein expression and enzyme activity in liver and kidney; protein levels were 2.1- and 1.8-fold higher in hepatic and renal cytosols of AAI pretreated mice than in control (untreated) mice, respectively. In contrast, no effect of AAI pretreatment was observed in mouse lung (Fig. 2A, *upper panel*). The increase in NQO1 protein expression in liver and kidney was paralleled by an increase in NQO1 activity, however, no enzyme activity was detectable in pulmonary cytosol isolated from AAI-pretreated mice (Fig. 2A, *lower panel*).

In contrast to these results, pretreatment of rats with AAI led to a significant increase in NQO1 protein expression only in kidney cytosol (1.4-fold, $p<0.05$); no increased expression was observed in cytosols isolated from liver and lung (Fig. 2B, *upper panel*). Likewise, a significant increase in NQO1 enzyme activity caused by AAI pretreatment was found only in rat kidney (1.5-fold, $p<0.001$) (Fig. 2B, *lower panel*).

3.3. AAI-DNA adduct formation mediated by mouse and rat cytosols

The efficiencies of hepatic, renal and pulmonary cytosols from AAI-pretreated mice and rats to activate AAI generating DNA adducts were compared with those from untreated animals (Fig. 3). AAI was metabolically activated in hepatic cytosolic samples of mice and rats as well as renal and pulmonary cytosols of rats only. Always the same pattern of AAI-DNA adducts was generated, consisting of three DNA adduct spots (see Fig. 1, *insert*). The same DNA adduct pattern by ^{32}P-postlabeling has been observed in renal tissue from AAN and BEN patients [4,10,11,14,15] and adducts were previously identified [2,11] as 7-(deoxyadenosin-N^6-yl)aristolactam I (dA-AAI), 7-(deoxyguanosin-N^2-yl)aristolactam I (dG-AAI) and 7-(deoxyadenosin-N^6-yl)aristolactam II (dA-AAII) (Fig. 1). No DNA adducts were observed in control incubations carried out in parallel without cytosols, or without DNA, or without AAI (data not shown).

Similar levels of AAI-DNA adducts were formed in *ex vivo* incubations using hepatic cytosols of mice and rats with AAI and DNA, and also with rat renal cytosols, and adduct levels practically corresponded to NQO1 activity in these cytosolic samples (compare Figs. 2 and 3). However, even though the highest NQO1 activity in rats was found in the rat lung cytosols (see Fig. 2B, *lower panel*), the lowest levels of AAI-DNA adducts were formed in these lung samples (Fig. 3B). Likewise, only background levels of AAI-DNA adducts were found in incubations containing mouse pulmonary and renal cytosols, and therefore were not quantified.

Higher AAI-DNA adduct levels (>1.7-fold, $p<0.01$) were formed by hepatic cytosols from AAI-pretreated mice and rats compared to those isolated from untreated animals (Fig. 3). A 2.5-fold ($p<0.001$) increase in AAI-DNA adduct formation was also found in renal cytosol isolated from AAI-pretreated rats relative to control (untreated) animals (Fig. 3B), while no effect of AAI pretreatment on DNA adduct formation was found in lung cytosol. The enhanced AAI-DNA adduct levels in mouse liver and rat kidney cytosols corresponded to higher cytosolic NQO1 protein levels in these cytosolic samples (compare Figs. 2 and 3). However, the increase in adduct levels in rat hepatic cytosol (>1.7-fold) was higher than the observed NQO1 protein expression (1.1-fold) and NQO1 enzyme activity in hepatic cytosol was even not increased by AAI-pretreatment of rats (Figs. 2 and 3). We can only speculate to explain this finding but another rat hepatic nitroreductase such as xanthine oxidase, which is also able to activate AAI [21-23], might be one of the reasons responsible for this phenomenon.

4. Discussion

Previously we found that AAI, in the presence of NADPH (a cofactor of NQO1), is activated by human liver and kidney cytosolic fractions, as well as purified human NQO1, to DNA adducts identical to those found in humans diagnosed with AAN and BEN [21-23,25,26]. These results suggested that NQO1 might be the principle enzyme responsible for AAI activation. In the present study we used Western blot analysis to determine NQO1 protein levels in cytosols from liver, kidney and lung of mice and rats, two animal models which are sensitive to the toxic and carcinogenic effects of AAI, and utilized in the AAN experimental protocols [2,21,22,35-38], NQO1 enzyme activity and AAI-DNA adduct levels were also determined. Furthermore, we evaluated not only basal NQO1 expression, but also NQO1 expression following AAI pretreatment. This study is a continuation of our previous work which investigated the expression of NQO1 in several genetically modified mouse lines [38] and aims to enhance our understanding on the role of NQO1 in AAI bioactivation.

Our results demonstrated that expression of NQO1 protein and enzyme activity was species- and organ-specific. In mice, cytosolic NQO1 protein levels and enzyme activity were the highest in kidney which is in line with the nephrotoxic properties of AA. In rats, however, the highest levels of NQO1 protein and NQO1 activity were found in lung, whereas they were the lowest in kidney.

Our study also indicated that pretreatment with AAI induces NQO1 protein levels and enzyme activity in mouse liver and kidney cytosol and in rat kidney cytosol. We found that increased NQO1 protein levels in kidney and liver correlated with NQO1 enzyme activity in these two organs. However, only in mouse liver cytosol, and cytosol of rat kidney, increases in the NQO1 protein levels also paralleled elevated levels of AAI-DNA adducts formed in *ex vivo* incubations of AAI and DNA with these cytosols. In contrast, the increase in AAI-DNA adduct levels formed in *ex vivo* incubations of rat hepatic cytosol with AAI was higher than

the elevated NQO1 protein expression and NQO1 enzyme activity in this cytosol was even not increased by treating rats with AAI. In this context it is noteworthy that previous studies have shown that xanthine oxidase is capable of activating AAI [21,22]. Thus, only the AAI-mediated induction of NQO1 in mouse liver and rat kidney enhances the reductive bioactivation of AAI to form DNA adducts, thereby enhancing its own genotoxic potential in these organs.

The near absence of significant AAI-DNA adduct formation detected from *ex vivo* incubation of AAI with mouse kidney and lung cytosolic fractions contrasted with results of corresponding experiments with rat systems. Moreover, the situation in mouse lung is completely different from that in the other two organs investigated and from that in rat lung. Basal and induced NQO1 protein levels in mouse lung, as measured by Western blotting, were similar to that in liver, but NQO1 enzyme activity was not detectable in lung after AAI pretreatment. Several reasons for this observation and low AAI-DNA adduct formation in *ex vivo* incubation of AAI with mouse lung and kidney cytosols are possible: (*i*) the expressed NQO1 protein in lung is inactive; (*ii*) menadione and AAI are not good substrates for lung NQO1; (*iii*) an as-yet unknown inhibitor is present in mouse lung and kidney cytosol, thereby decreasing reduction of menadione and/or AAI; or (*iv*) pulmonary and renal NQO1 proteins have undergone allosteric effects due to interactions with different substrates including menadione or AAI. Which of these reasons might be most important in our experiments is difficult to estimate, but it is noticeable that no NQO1 enzyme activity was seen in the mouse lung after AAI pretreatment [38]. Further, also in rat lung cytosols some discrepancies in NQO1 expression and its activity remain to be explained. Even though NQO1 protein expression in lung cytosols both from untreated as well as AAI-pretreated rats correlated well with NQO1 enzyme activity, low efficiencies of these lung cytosols to catalyze AAI-induced DNA adduct formation were found.

The differences in NQO1 protein expression, its enzyme activity and reductive activation of AAI in individual tested organs of mice and rats suggest the potentially different metabolism, nephrotoxicity and carcinogenicity patterns of AAI in these animal models. This suggestion is supported by several former studies; different responses of mice and rats to AAI to mediate development of interstitial nephropathy and associated cancer of the upper urinary tract as well as biotransformation of AAI were found previously [22,24,36,44-50].

It is noteworthy that the increase in NQO1 protein expression and enzyme activity due to AAI pretreatment was lower in rats than in mice. It is likely that this is caused by the different dosing protocols used for AAI treatment in rats and mice; not only the administered dose of AAI was different (20 versus 50 mg/kg body weights in rat and mice) but also the route of AAI administration (*i.p.* to rats *vs* gavage to mice). As exposure route was different for the two animal models, toxicokinetics are most probably different between the models and a direct comparison is thus not possible.

The highest levels of NQO1 protein and enzyme activity in mouse kidney and the efficient induction of this enzyme by AAI in this organ are consistent with the finding that AAI-DNA adduct formation was the highest in mouse kidney *in vivo* [27,29,30]. In other words, AAI can induce NQO1 in kidney, which is the target organ of AAI-induced toxicity, and increased DNA adduct formation contributes to the observed toxicity in this organ which is less pronounced in the other organs of mice exposed to AAI [27,29,30]. The stimulating effects of AAI pretreatment on NQO1 induction found here in mice and rats also confirmed results of previous studies [38,51,52]. Therefore this enzyme is likely to be induced in the kidneys of AAN and BEN patients, which may contribute to their increased risk for urothelial cancer.

Beside NQO1, the CYP1A1/2 enzymes were found to be included into AAI metabolism [27-33]. Using *Cyp1a*-knock-out (single and double knock-outs) and *CYP1A*-humanized

mouse lines, the crucial role of CYP1A1 and 1A2 enzymes in AAI metabolism *in vivo* was unambiguously proven [29,30,36]. Human and rodent CYP1A1 and 1A2 play a dual role in the metabolism of AAI. Under anaerobic conditions they reductively activate AAI, while under oxidative conditions they are the predominant enzymes catalyzing *O*-demethylation of AAI to AAIa (*i.e.* detoxication). This AAI oxidation finally leads to a decrease in AAI-induced renal injury. Based on current knowledge we propose that AAI metabolism *in vivo* is determined by the binding affinity of AAI to CYP1A1/2 or NQO1, and their enzymatic turnover as well as by the oxygen levels in the organs [53]. However, the extent to which these enzymes contribute to AAI-mediated nephropathy and upper urothelial tract carcinoma in humans is still a matter of debate and remains to be investigated. Unfortunately, studies investigating a possible association between genetic polymorphisms of enzymes metabolizing AAI with the risk of developing AAN/BEN and/or upper urothelial tract carcinoma have lead to controversial results. It was reported that polymorphisms in the human *NQO1* gene are important in AA-induced nephropathy [54,55]. The *NQO1*2/*2* genotype (*NQO1 C609T* polymorphism), resulting in very low NQO1 expression levels, has been shown to predispose BEN patients to a much higher incidence of urothelial cancer (OR=13.75, 95%CI 1.17-166.21) [54,55]. This finding appears to be opposite to what one might expect, given our demonstration herein of the importance of NQO1 in AAI activation; however, diminished NQO1 metabolism of AAI could lead to an enhanced body burden which might lead to increased risk of tumorigenesis over time in BEN patients [38]. However, no significant association was found between this *NQO1 C609T* polymorphism and the risk of developing AAN [56]. This discrepancy shows that the development of the nephropathies (AAN and BEN) and upper urothelial tract carcinoma by AAI seems to follow different paths. Maybe AAI as such is nephrotoxic, but reductive activation, *e.g.* catalyzed by NQO1 is needed for genotoxicity leading to cancer.

In contrast to NQO1, even thought the *CYP1A1* and *CYP1A2* genes are also polymorphic [57-60], no relationships between the polymorphisms of CYP1A1 and AA-induced nephropathy were found in AAN/BEN patients [55,56] and the changes in the *CYP1A2* gene have not been investigated as yet.

5. Conclusions

Utilizing Western blot analysis NQO1 protein levels were analyzed in liver, kidney and lung of untreated and AAI-pretreated mice and rats. Our study demonstrated that AAI has the potential to induce the activity of the cytosolic nitroreductase NQO1 in liver and kidney in both animal models. Our studies and the findings of others [54,55] indicate that certain *NQO1* genotypes appear to be linked to an increased risk of urothelial cancer in BEN patients, underscoring the potential clinical importance of NQO1 activity in AAI-exposed humans. However, because the studies evaluating association of genetic polymorphisms of the enzymes metabolizing AAI with a risk of developing AAN, BEN and upper urinary tract urothelial carcinoma have brought controversial results, another approach should be utilized to evaluate the contribution of the enzymes metabolizing AAI in these processes. We propose that analyses of the expression levels of these enzymes (NQO1, CYP1A1 and 1A2) and their phenotyping in AAN and BEN patients should bring more valuable data on their real contribution to the development of AA-induced nephropathies and cancer risk among these patients.

Conflict of interest statement

The authors declare that there are no conflicts of interest to this work.

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Legends to Figures

Fig. 1. Pathways of biotransformation and DNA adduct formation of AAI. dA-AAI, 7-(deoxyadenosin-N^6-yl)aristolactam I; dG-AAI, 7-(deoxyguanosin-N^2-yl)aristolactam I; NR, nitro-reduction; UGT, UDP glucuronosyltransferase; SULT, sulfotransferase. Insert: Autoradiographic profile of AAI-DNA adducts formed by incubation of AAI with hepatic cytosol from mice, using the nuclease P1 enrichment version of the ^{32}P-postlabeling assay. The adduct profile shown is representative of those obtained in cytosolic fractions of other organs of mice and rats analyzed.

Fig. 2. NQO1 protein expression (blue columns) and NQO1 enzyme activity (red columns) in cytosols isolated from liver, kidney and lung of mice (A) and rats (B). Mice were pretreated with a single oral dose of 50 mg/kg body weight AAI or left untreated (control). Rats were pretreated with a single *i.p.* dose of 20 mg/kg body weight AAI or left untreated (control). NQO1 protein expression in cytosols (upper panel) was determined by Western blotting (see insert). Human recombinant NQO1 was used to identify the mouse and rat NQO1 band in murine and rat cytosols, respectively (data not shown). NQO1 enzymatic activity in cytosols (lower panel) was determined as described in chapter 2.4. All values are given as means ± S.E.D. (n = 4). ND - not detected. Comparison was performed by t-test analysis; $*p < 0.05$, $***p < 0.001$, different from control.

Fig. 3. DNA adduct formation *ex vivo* by AAI in mouse (A) and rat cytosols (B) determined by ^{32}P-postlabelling. Mice were pretreated with a single oral dose of 50 mg/kg body weight AAI or left untreated (control). Rats were pretreated with a single *i.p.* dose of 20 mg/kg body weight AAI or left untreated (control). Values are given as the means ± S.E.D. (n = 4). RAL, relative adduct labeling. ND, not determined (levels of AAI-DNA adducts were at the

detection limit). Comparison was performed by *t*-test analysis; $**p < 0.01$, $***p < 0.001$, different from control.

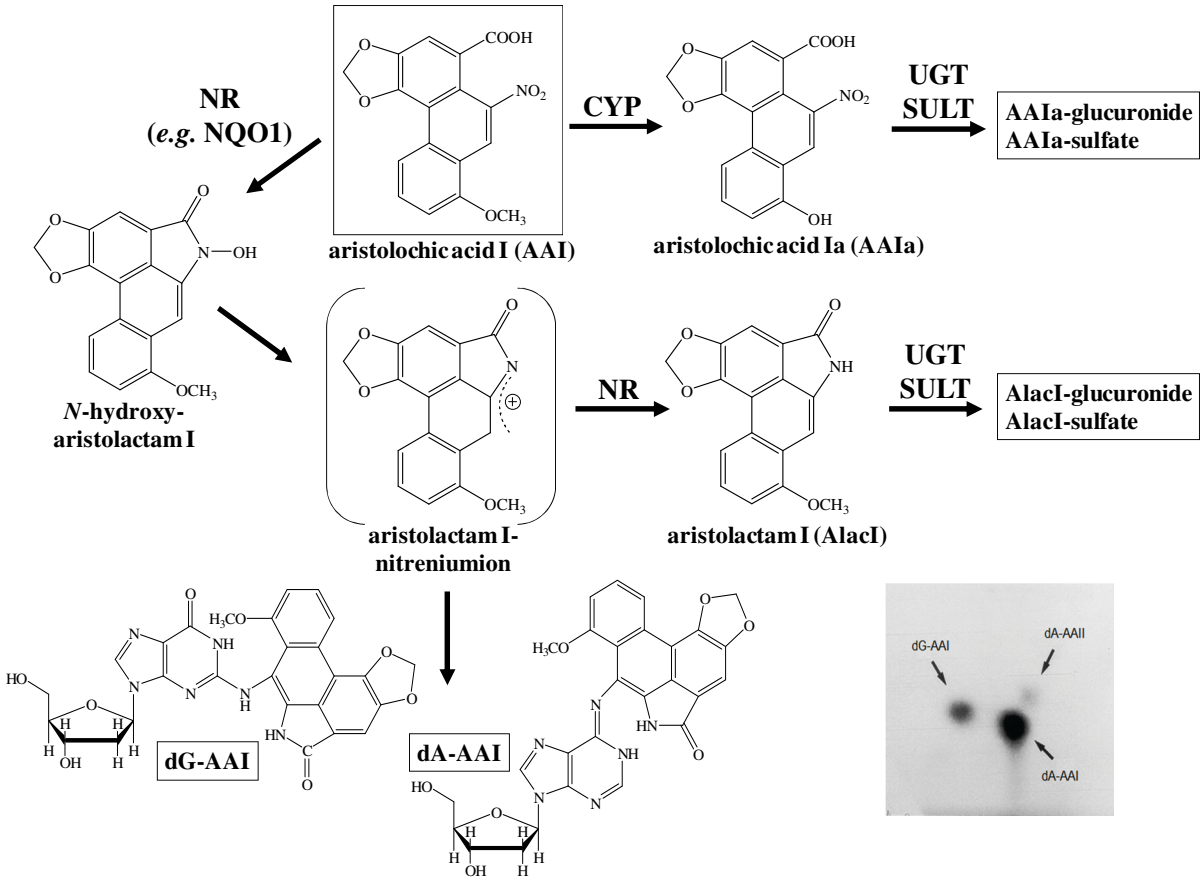
NR
(e.g. NQO1)
aristolochic acid I (AAI)
CYP
aristolochic acid Ia (AAIa)
UGT
SULT
AAIa-glucuronide
AAIa-sulfate
N-hydroxy-
aristolactam I
aristolactam I-
nitreniumion
NR
aristolactam I (AlacI)
UGT
SULT
AlacI-glucuronide
AlacI-sulfate
dG-AAI
dA-AAI
dG-AAI
dA-AAII
dA-AAI

Figure 1

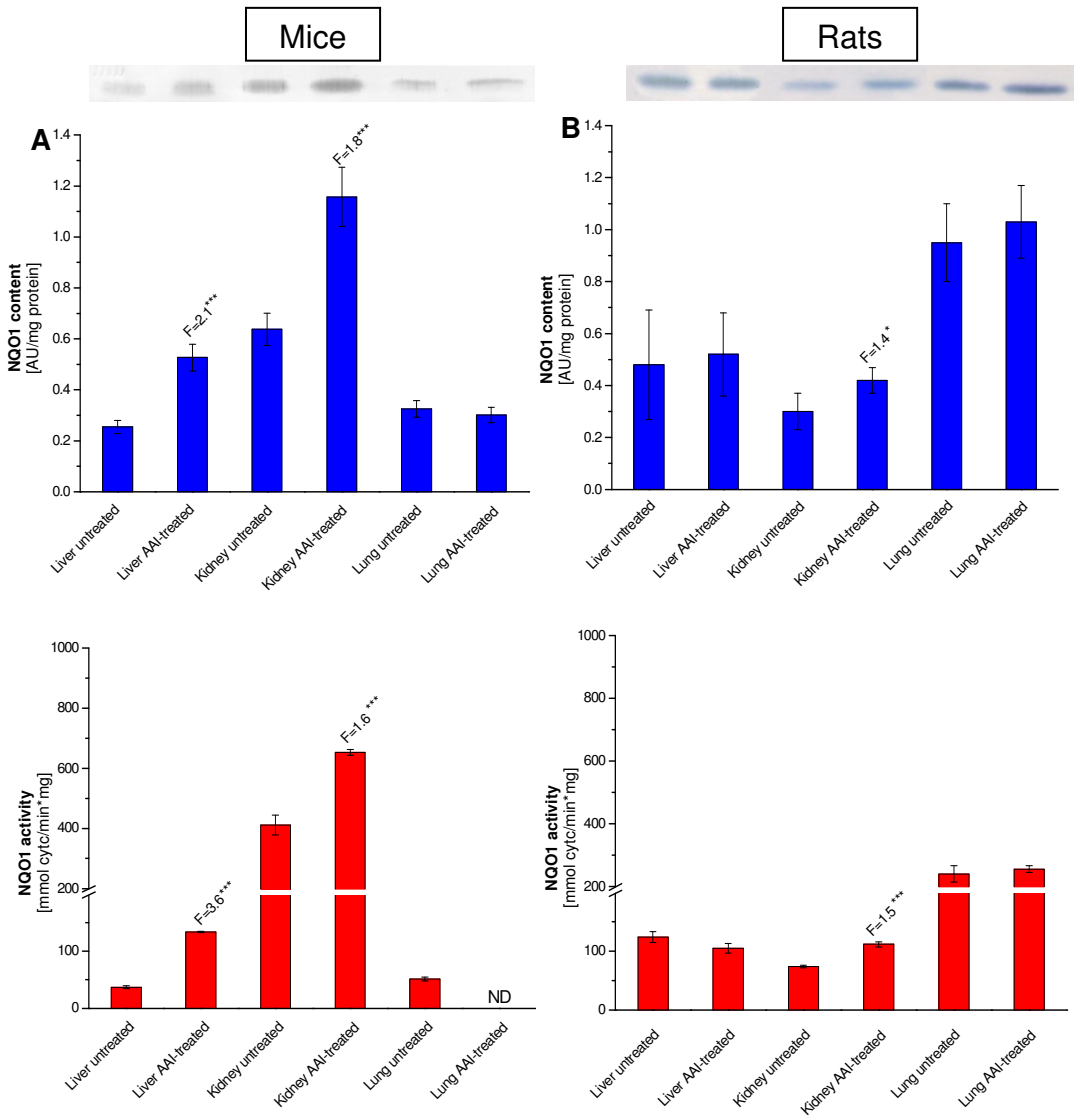
Mice
Rats
A
B
NQO1 content
[AU/mg protein]
F=2.1***
F=1.8***
F=1.4*
NQO1 activity
[mmol cytc/min*mg]
F=3.6***
F=1.6***
F=1.5***
ND
Liver untreated
Liver AAI-treated
Kidney untreated
Kidney AAI-treated
Lung untreated
Lung AAI-treated

Figure 2

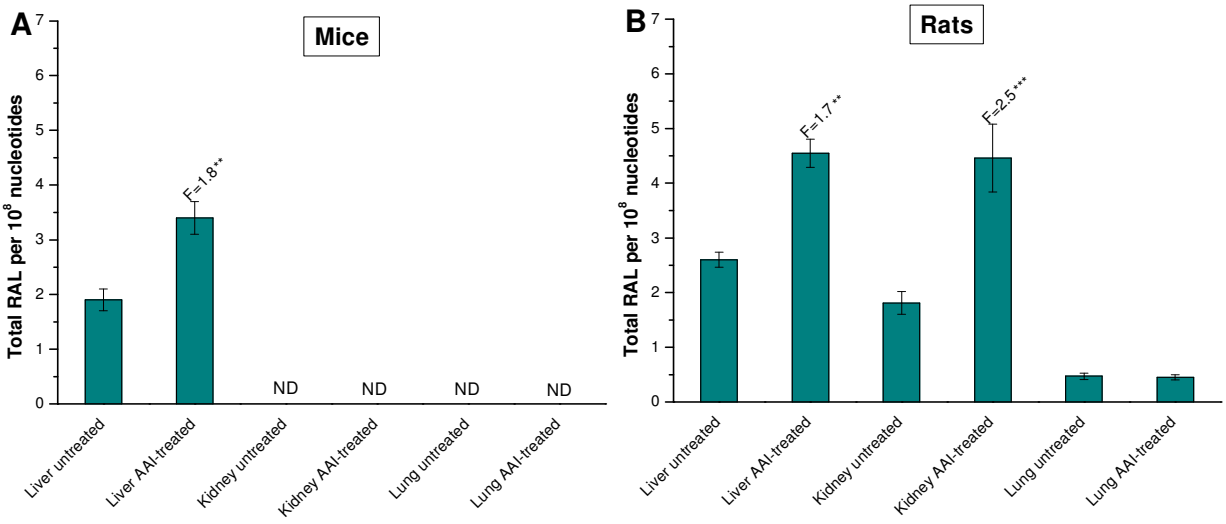
A
Mice
Total RAL per 10^8 nucleotides
F=1.8**
ND
ND
ND
ND
Liver untreated
Liver AAI-treated
Kidney untreated
Kidney AAI-treated
Lung untreated
Lung AAI-treated
B
Rats
Total RAL per 10^8 nucleotides
F=1.7**
F=2.5***
Liver untreated
Liver AAI-treated
Kidney untreated
Kidney AAI-treated
Lung untreated
Lung AAI-treated

Figure 3